CHAPTER XV

EGG, SPERM, FERTILIZATION, AND CLEAVAGE

As early as 6 to 10 weeks after setting, young *C. virginica* of New England waters, then 6 to 8 mm. in height, develop primordial gonads of profusely branching tubules (Coe, 1932a). At this stage the germinal epithelium is a layer of morphologically undifferentiated cells; some of them will transform into larger cells to become ovocytes, i.e., the cells destined to develop into mature eggs. The smaller cells of the epithelium proliferate very rapidly and are recognizable as the male germ line, and eventually develop into spermatozoa. For several weeks the immature, or primary, gonad of an oyster remains nonfunctional and bisexual (ambisexual), for it contains both male and female germ cells which will transform into mature spermatozoa or ova during the following summer. In some individuals the primary bisexual gonad is retained until the second year, a delay which Coe (1932a, 1938) attributes to poor nutrition.

The more rapid multiplication of male germ cells suppresses the development of ovocytes and results in a predominance of males among the 1-year-old oysters and in the appearance of different degrees of intersexuality (predominance of the cells of one sex over the other). In the same brood which contains also distinctly ambisexual oysters there are, however, other young individuals in which the primary gonad develops directly into ovary or spermary. Local conditions on oyster beds apparently influence the tempo of changes. In the warmer waters at Beaufort, N.C., young oysters are more apt to develop directly into females than in the northern cold waters of New England. Coe (1938) found that the proportion of females to 100 males varied at the first breeding season between 37.1 and 48.8 at Beaufort; 5.6 and 24 at Milford, Conn.; and 3.3 and 12.5 at New Haven Harbor. The differences are not consistent with geographical latitude since the female to male ratio at West Sayville, Long Island, N.Y., was 31.2; at Delaware Bay 41.9; and at Apalachicola, Fla., 7.1. It is obvious that these variations cannot be attributed to temperature alone and are probably caused by a combined effect of environmental conditions.

Toward the end of the second breeding season the primary gonad is transformed into a definite ovary or spermary (fig. 291). The gametogenesis, i.e., complete transformation of the primordial germ cells into mature ova (ovogenesis) or spermatozoa (spermatogenesis), is a very complex process. The differentiation is accompanied by rapid multiplication of the new generations of cells which

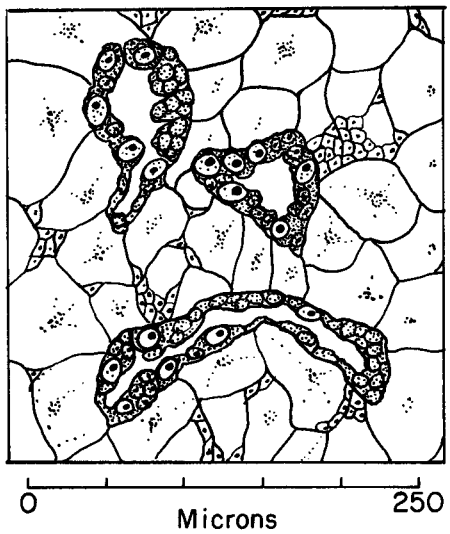

FIGURE 291.—Section of a gonad of *C. virginica* at an early stage of differentiation. End of March, Woods Hole, Mass. The larger, clear cells are ovogonia, the smaller ones are undifferentiated cells of germinal epithelium. Bouin, hematoxylin-eosin.

extend inward and fill up the lumen of the follicles. Early at this stage the sex cells become dense and opaque, a condition which interferes with cytological study.

Gametogenesis of *C. virginica* and *O. lurida* has been studied by Coe (1932a, 1932b, 1934, 1936, 1938) and that of the Australian rock oyster, *C. commercialis*, by Cleland (1947). Only the main points of this process were disclosed by these investigations.

OVOGENESIS

In all animals the primordial germ cells become distinguishable as primary ovogonia in the females or spermatogonia in the males after a certain number of divisions. After a period of quiescence they begin to divide again and give rise to secondary ovogonia or spermatogonia. After several generations the cells stop dividing and enter a growth period, which is more prolonged in the females than in the males. The growth period is characterized by a series of cytological changes, each differing from the preceding stage. The cells which will produce gametes are at this stage called auxocytes, from the Greek "auxesis" meaning growth, and are referred to as ovocytes in the female and spermatocytes in the male.

Ovogenesis in oysters begins with the appearance of enlarged cells in the germinal epithelium. These are the ovogonia, which in *C. virginica* and *O. lurida* are distinguished from other cells of the germinal epithelium by their relatively large nuclei with conspicuous nucleoli and loose chromatin network (fig. 292, og). The ovogonia usually lie next to the follicle wall, and their distal sides do not protrude into the lumen. Differences between the early ovogonia and indifferent residual cells (I) are not conspicuous. Examination of a series of sections and study of the sequence of changes in the appearance and structure of the cells are necessary to assure a positive identification.

After one or two divisions the ovogonia change in appearance as well as in size. This generation of female sex cells called ovocytes can be recognized by the presence of fibrillar mitochondrial bodies (sometimes called yolk nuclei), and by the spiremes of densely packed chromosomes (figs. 293 and 294). Their nucleoli become very conspicuous.

During the last stage of ovogenesis the ovocyte begins to grow rapidly, and the distal part, grossly enlarged and rounded, protrudes into the lumen of a follicle. At the same time the connection

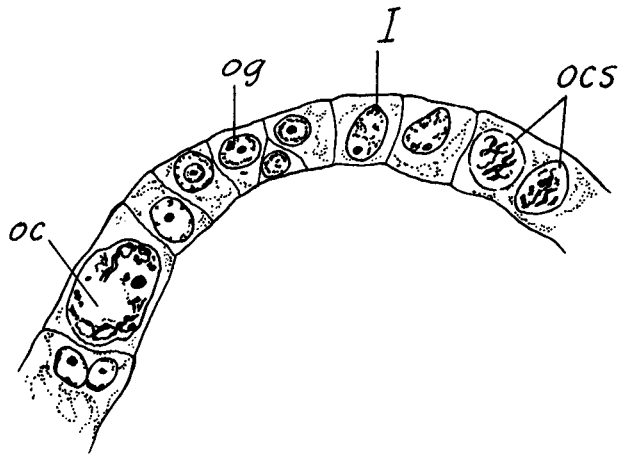

FIGURE 292.—Follicle wall of the ovary of *C. virginica*. I—indifferent residual cell; oc—residual ovocyte; ocs—two young ovocytes in synaptic phase; og—group of ovogonia. Redrawn from Coe, 1932a, fig. 9. Highly magnified.

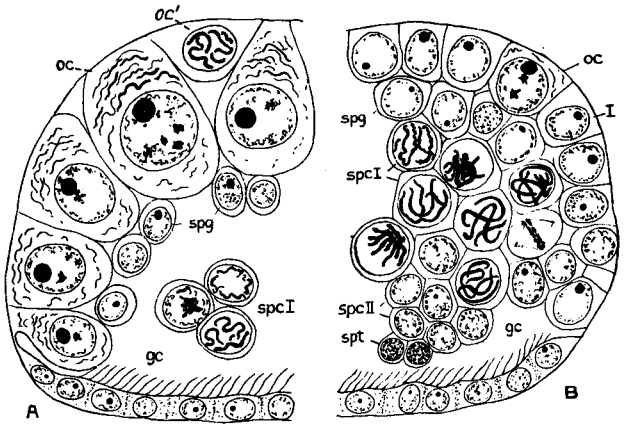

FIGURE 293.—Portions of two follicles of a bisexual gonad of 4-month-old *C. virginica*. A—predominantly female, and B—predominantly male follicle; gc—genital canal lined with ciliated cells; oc—large ovocyte; oc'—young ovocyte in spireme phase; spcI—primary spermatocytes in spireme phase; spcII—secondary spermatocytes; spt—spermatides. Photographically reproduced from Coe, 1932a, fig. 6. Highly magnified.

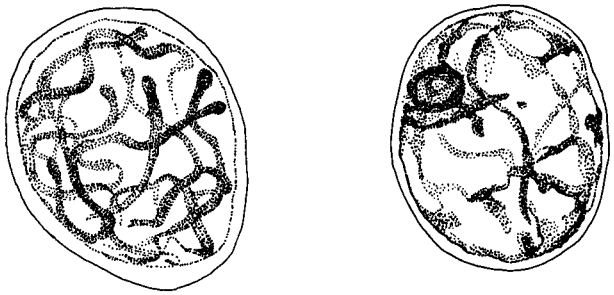

FIGURE 294.—Two young ovocytes at spireme stage in a mature ovary of *C. virginica*. Redrawn from Coe, 1932a, fig. 9. Highly magnified.

with the basal membrane of the follicle wall is narrowed to an elongated stem. The nucleus increases greatly in bulk, and the developing egg assumes a pear-shaped form. An accumulation of dark granules (mitochondria) at the proximal end of the cells may indicate that food for the growing ovocyte is obtained through the wall of the follicle. The granules are not pronounced in the ovocyte of *C. virginica* but are conspicuous in some other bivalves, particularly in *Sphaerium* (Woods, 1932). From the beginning of sexual differentiation to the final maturity of an ovum, the early ovocyte increases in volume more than 3,000 times.

Ovogenesis in the Sydney rock oyster *C. commercialis*, described by Cleland (1947), is somewhat different from the ovogenesis of the American species. At the earliest stage before the start of the growth phase an ovocyte of the rock oyster is a small cell, 4 μ to 5 μ in diameter. Two-thirds of the cell is occupied by a clump of chromosomes surrounded by a rim of clear cytoplasm. Cleland identifies this stage as a definite auxocyte, i.e., an ovocyte just before entry into the growth phase. At ths stage the cell has no nucleolus.

The definite auxocyte begins to grow and passes through three stages (called by Cleland Auxocyte I, II, and III) which differ in size and nuclear structure. Auxocyte I has a diameter of about 9 μ, with a relatively large germinal vesicle (6 μ) and a nucleolus of about 1.7 μ. The nucleus is centrally placed in the homogeneous cytoplasm with an excentric nucleolus which is not in contact with the nuclear membrane. Auxocyte II has diameter of 12.6 μ, with the germinal vesicle (nucleus) about 7 μ and nucleolus 2 μ to 3 μ. The cell is usually spherical with a centrally located germinal vesicle and chromosomes spaced more widely than in Auxocyte I. At this stage a group of granules appears at one pole of the nucleus. Auxocyte III is a spherical cell 20 μ in diameter, with the germinal vesicle measuring 11 μ and eccentrically located nucleolus of about 4.2 μ in diameter. The cell is separated from the wall and is free in the lumen of a follicle. Protein granules are abundant along the periphery of the cell where they are found in a mature egg. The mature ovocyte of *C. commercialis* has a diameter of about 38 μ. The nucleus is large, about 21 μ across; the nucleolus is 4.6 μ. The chromosomes are paired and are usually placed peripherally in the germinal vesicle but not in contact with the nuclear membrane. Crossing-over is frequently seen at this stage but the chromosomes are not coiled.

SPERMATOGENESIS

Spermatogenesis in the oyster is known primarily from the studies by Coe (1931) on the development of the gonad of young *O. lurida*. Comparison with the gonads of *C. virginica* shows that there is close agreement in the general features of the process in both species. Progressive stages of the formation of sperm begin with the undifferentiated gonia which line the inner wall of the gonad follicles. After a large number of descendants have been produced the spermatogonia can be distinguished from the ovogonia by their smaller size and position within the follicles. Ovogonia lie in a single row along the wall; the primary spermatogonia of *C. virginica* are found either singly or in groups between the ovogonia lining the wall and in the lumen (fig. 295).

In the hermaphroditic gonad of *O. lurida* a single primary spermatogonium divides several times to form a cluster of cells which become separated from the follicle wall and occupy a position toward the center of the lumen (fig. 296).

The number of divisions of spermatogonia presumably depends on the amount of nourishment available to the gonad. Coe estimates that in *O. lurida* each primary spermatogonium divides six to nine times to produce a cluster of 64 to 500 cells. In spite of the close contact the adjacent cells of the clusters are separate but are held together by a delicate noncellular secretion. Its

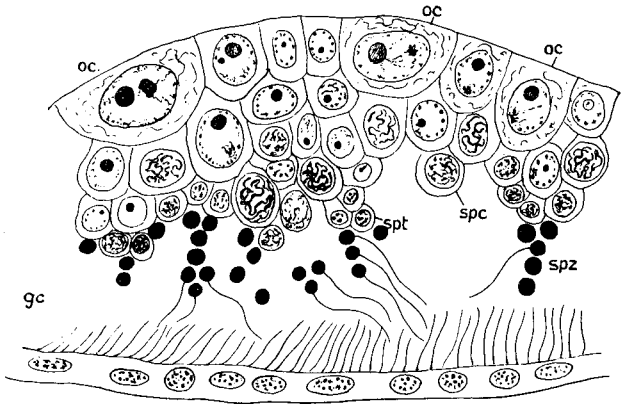

FIGURE 295.—Portion of bisexual gonad of young *C. virginica*. gc—genital canal; oc—ovocytes with spermatogonia filling the lumen; spc—spermatocytes; spt—spermatids; spz—spermatozoa. Photographically reproduced from Coe, 1934, fig. 5A. Highly magnified.

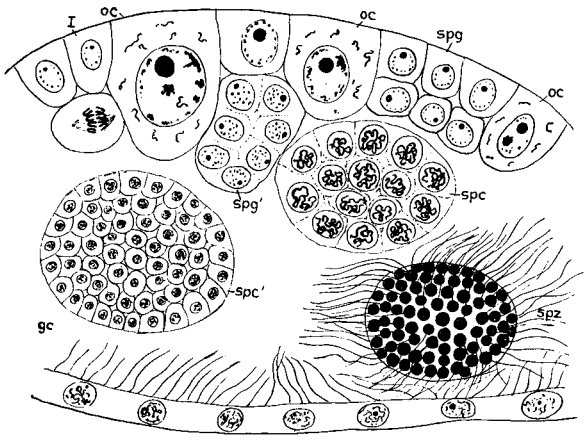

FIGURE 296.—Portion of an hermaphroditic gonad of *O. lurida*. gc—genital canal; I—indifferent cells; oc—ovocytes; spc—primary spermatocytes; spc¹—secondary spermatocytes; spz—spermatozoa united into a sperm ball. Photographically reproduced from Coe, 1934, fig. 5B. Highly magnified.

chemical nature has not yet been determined. In poorly preserved preparations the clusters sometimes have the appearance of syncytia with nuclei embedded in a common matrix. In both species all spermatogonia have a conspicuous nucleolus and loose chromatin reticulum. As the divisions proceed the diameter of the spermatogonia diminishes from about 6μ to 3μ or less at the last stage leading to the formation of primary spermatocytes. In *C. virginica* these cells are globular, each with a large nucleus resolved into slender threads (spiremes). This leptonema stage is frequently observed in the developing spermary but the conjugation of chromosomes (synapsis) has not been described with any detail. However, in reference to the spermatogenesis in *O. lurida*, Coe (1931) states that the leptotene stage "is followed by the usual process of synapsis."

The appearance of secondary spermatocytes is similar to that of the primary. In *C. virginica* they can be distinguished by their radial orientation in the lumen and small size. Different phases of spermatogenesis in *C. virginica* are shown in a semidiagrammatic drawing published by Coe (1932a) and reproduced in fig. 297. Meiotic divisions and the transformation of spermatids into mature spermatozoa have not been fully described for *C. virginica*. In a mature spermary the spermatozoa are always oriented with their tails toward the center of the lumen. The photomicrograph shown in fig. 298 shows the gradual increase in the number of male sex cells from the wall of the follicle toward the center. Successive stages of the spermatogenesis of *O. lurida* drawn by Coe are shown in fig. 299.

Secondary spermatocytes of *O. lurida* are held together in spherical masses. Close contact by the spermatids continues during their transformation into spermatozoa; in the sperm ball of a mature oyster the tails radiate from the center. Each sperm ball is composed of from 200 to 2,000 spermatozoa originating from a single spermatogonium (Coe, 1932b). During mitotic divisions the "prophase, metaphase, and telophase are all of typical appearance, with a delicate spindle of the usual form" (Coe, 1931). Because of the crowded condition of the metaphase and anaphase plates, Coe was unable to determine the chromosome number which he states "is not very large." In two diagrammatic drawings of spermatocyte division Coe (1931, fig. 3, E and F) figures 10 chromosome pairs. This is the most common number of chromosomes found by Cleland at the two- and four-cell stage of cleavage in the fertilized egg of *C. commercialis* (Cleland, 1947). The number of chromosomes seen during the cleavage of *C. virginica* eggs is discussed later (p. 345).

STRUCTURE OF THE MATURE EGG

Eggs in the mature ovary of *C. virginica* are pear-shaped and compressed. Many of them are attached to the follicle wall by long, slender peduncles; others are free in the lumen ready to be moved to the genital canals and discharged (fig. 300). The long axis of the eggs varies from 55μ to 75μ depending on their shape; the width at the broadest part measures from 35μ to 55μ, and the diameter of the nucleus is from 25μ to 40μ. The oblong shape is retained for some time after the discharge of eggs into water but gradually the egg becomes globular and denser. Under the transmitted light of a microscope the nucleus appears as a large, transparent area surrounded by densely packed granules (fig. 301). In a globular egg the nucleus cannot be seen unless it is cleared in glycerol or other clarifying reagents (fig. 302).

Eggs of oysters living under marginal conditions in water of salinity less than 10 °/₀₀ frequently become cytolyzed upon their removal from the ovary; the nuclei appear larger than those of normal eggs. Only 1 or 2 percent of these eggs is fertilizable. The delicate primary or "vitelline" membrane surrounding the unfertilized egg is

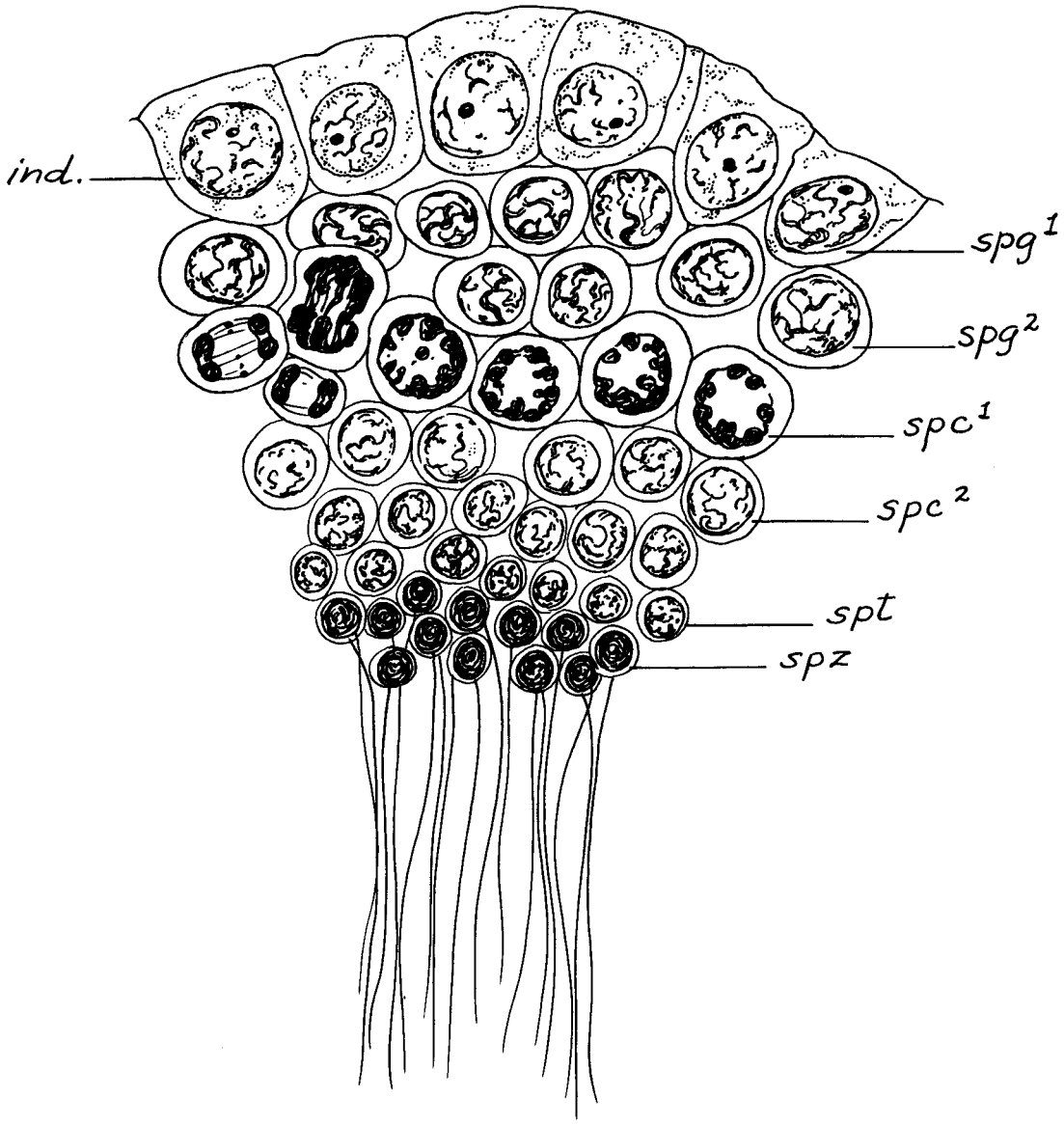

FIGURE 297.—Mature spermary of *C. virginica*. ind—indifferent cells; spg¹ and spg²—primary and secondary spermatocytes; spt—spermatids; spz—mature spermatozoa. Redrawn from Coe, 1932a, fig. 8.

secreted by the egg itself while it is still in the ovary. Raven (1958) states that in some cases the vitelline membranes of *Ostrea*, *Mytilus*, *Dreissensia*, and *Dentalium* are thrown off soon after shedding. I have not seen this happen in the eggs of *C. virginica*.

CYTOPLASMIC INCLUSIONS

Cytoplasmic components of an oyster egg are not well known primarily because the ultrastructure has not been studied by electron microscopy. Certain types of minute granules can be seen, however, in examination of live eggs under high magnification of phase contrast lenses; by applying vital and metachromatic stains; by centrifuging whole eggs or their homogenates in order to separate various components and study their staining reactions. For descriptions of the techniques used in modern cytology the reader is referred to the textbooks on cytology and microscopic histochemistry (Gomori, 1952; DeRobertis, Nowinski, and Saez, 1960; and others). The yolk constitutes the major part of the eggs of marine bivalves. Quantitative data on the amount of yolk in oyster eggs are lacking, but for *Cumingia tellinoides* and *Mytilus californianus* Costello (1939) found that

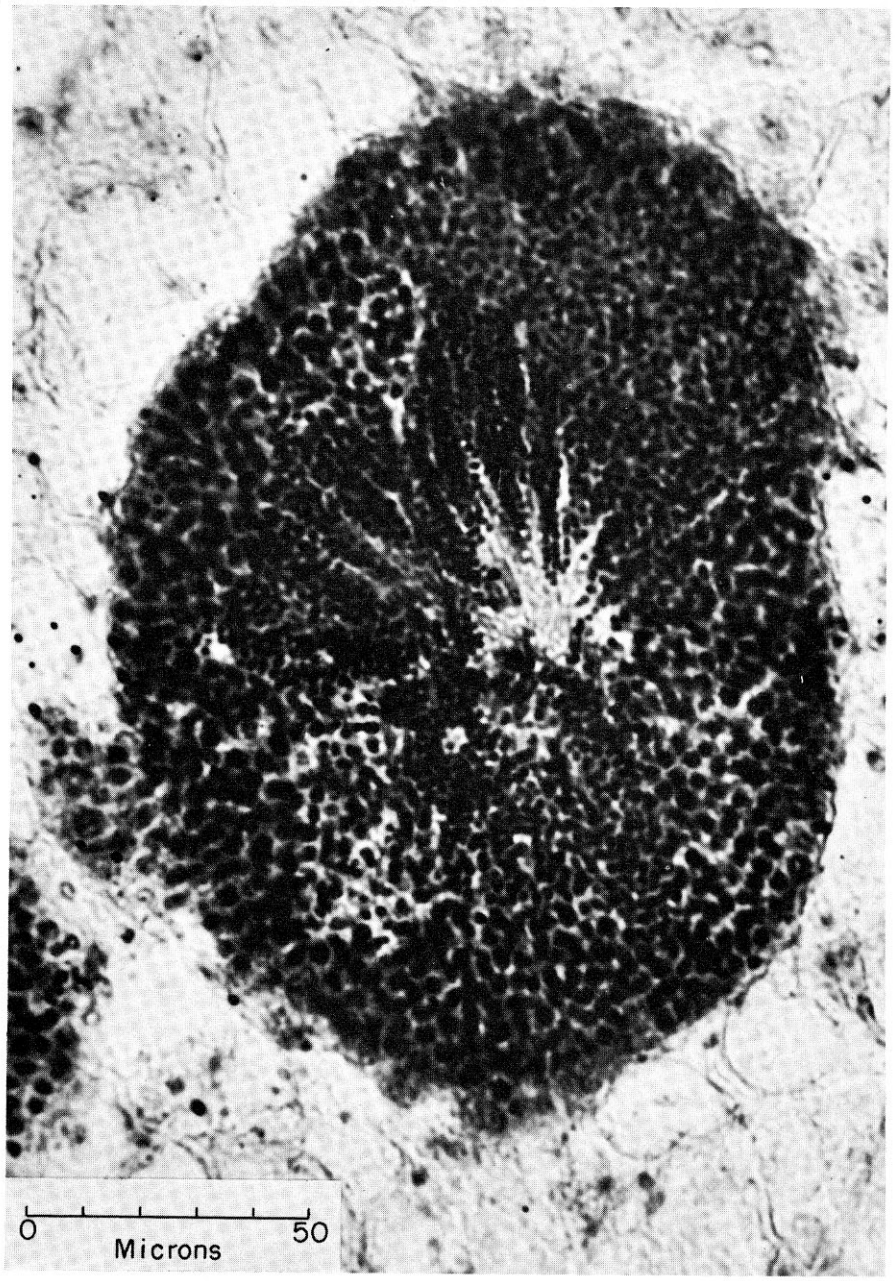

FIGURE 298.—Photomicrograph of a cross section of one follicle of a fully mature spermary of *C. virginica*. Hematoxylin-eosin.

yolk forms 35 and 31 percent respectively of the total egg volume. The estimates were obtained after a centrifugal force of 20,000 (*Cumingia*) and 4,800 (*Mytilus*) times gravity had been applied to the unfertilized eggs. In the cytoplasm of the eggs of the two species the relative volumes of hyaline zone were 42 and 55 percent and of the oil 10 and 14 percent.

The yolk of molluscan eggs is made of two types of granules, one of proteid and the other of fatty materials. In cytological literature the distinction between the proteid yolk and fatty yolk is not always made clear. In descriptions of the cytoplasmic inclusions of an egg based on light microscopy some authors apply the term exclusively to protein granules, while others, including Gatenby (1919), Gatenby and Woodger (1920), and Brambell (1924) in their studies of the

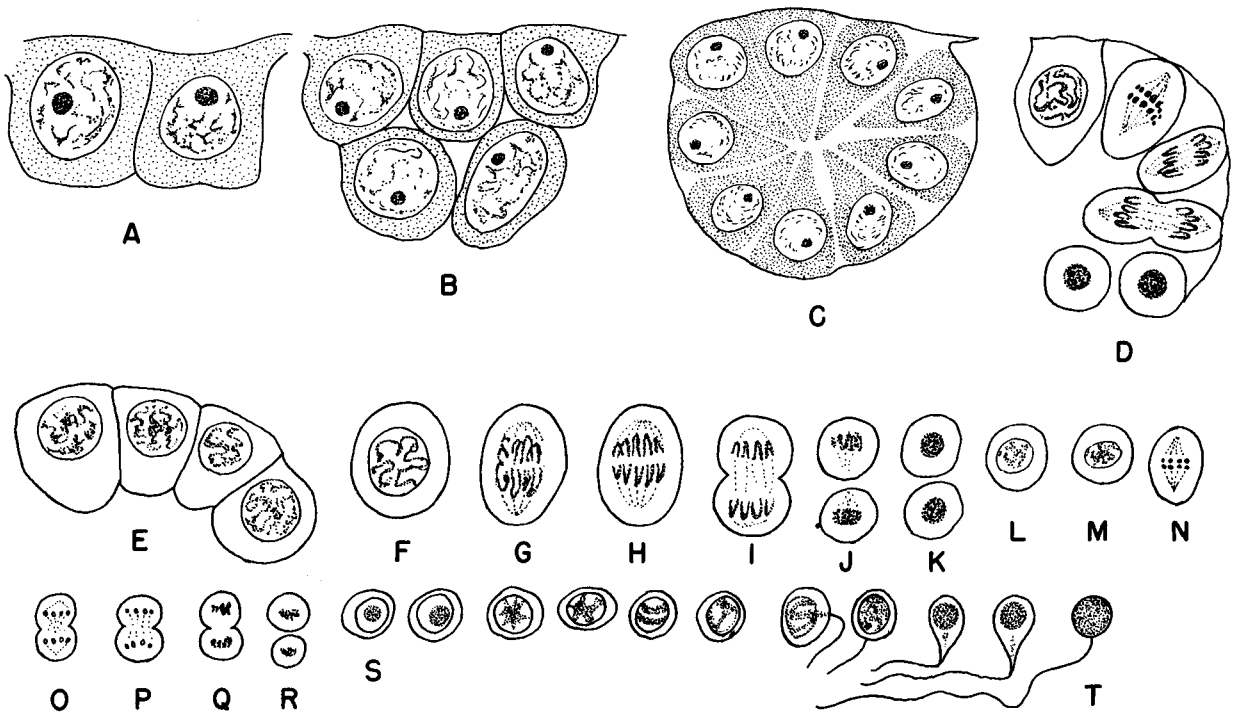

FIGURE 299.—Diagram of successive stages of spermatogenesis in *O. lurida*. A—two indifferent germ cells on the wall of the gonad; B—small group of spermatogonia, with reticular chromatin and conspicuous nucleoli; C—small group of secondary spermatogonia; D—division of secondary spermatogonia to form spermatocytes; E—primary spermatocytes with slender chromosomes; F to K—division of primary spermatocytes; L to R—division of secondary spermatocyte; S to T—transformation of spermatid into the mature spermatozoon. Redrawn from Coe, 1931, fig. 2.

gametogenesis in the gastropods *Helix*, *Limnaea*, and *Patella*, identify the protein granules as "mitochondria" and restrict the term yolk to fatty inclusions.

The role of the mitochondria in the formation of yolk has not been fully resolved. According to Rai (1930), the fatty yolk in the eggs of *C. cucullata* is formed directly from the Golgi vesicles as it is in ascidians, *Helix*, and other invertebrates; mitochondria do not participate in the vitellogenesis, and albuminous yolk is absent in the egg of the Indian oyster (*C. cucullata*). This view is in agreement with the conclusion of Worley (1944), who found no protein yolk in the eggs of *Mytilus* and *Ostrea*. The question is not settled because apparently the cytologists have no clear agreement on the difference between the protein yolk and mitochondria.

A study of cytoplasmic inclusions was made by Cleland (1947, 1951), who separated the granules found in egg cytoplasm of the oyster by differential centrifugation following homogenization. Mature eggs were suspended in a solution of 0.2 M potassium chloride and 0.02 N sodium citrate buffered to pH 7.5. Homogenates were obtained by blending the suspension in an electric blender surrounded by an ice jacket. By centrifuging the samples of homogenates at different speeds the following types of granules were obtained: P granules or protein yolk; L granules or lipid yolk; M granules or mitochondria; and S or submicroscopic granules. The P granules obtained by Cleland's technique are spherical and can be stained by Janus green B in the test tube. In the living egg these granules are located along the periphery and absorb Nile blue stain. After being centrifuged at 5,000 times gravity for 5 minutes they form a thick centrifugal layer with a sharp upper boundary. Alpha or lipid yolk granules are also spherical. They occupy the central part of the living egg. In the centrifuged egg they form a centripetal layer with a sharp lower boundary. Alpha granules of phospholipid and neutral fat can be recovered from the supernatants of homogenate suspensions. M granules or mitochondria in the live centrifuged egg form a thin, rather loose layer above the P granules and stain both with Janus green B and Nile blue.

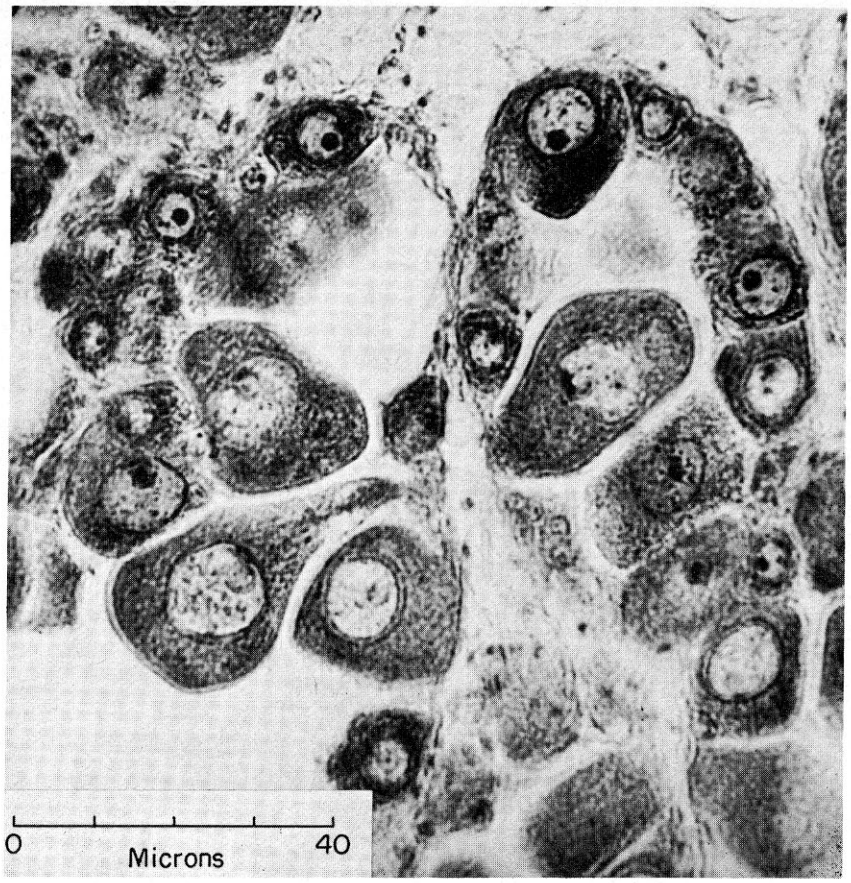

FIGURE 300.—Photomicrograph of eggs in the follicles of the ovary of *C. virginica* at the beginning of the spawning season. Ovocytes and small indifferent cells line the wall; mature eggs are either free or connected to the wall with long peduncles. Kahle, hematoxylin-eosin.

Cleland states that in the uncentrifuged mature egg they are unrecognizable. From homogenates the M granules can be separated by centrifuging for 10 minutes at 10,000 times gravity. Cytoplasm also contains submicroscopic or S granules (according to Cleland's terminology), which are separable by applying a centrifugal force of 20,000 times gravity for 30 minutes. These S granules are probably homologous to mammalian microsomes, i.e., the submicroscopic ribonucleoprotein particles which are considered to be the major sites of protein synthesis (a discussion of this problem is found in Shaver, 1957, and Novikoff, 1961b).

With the exception of pure lipid granules, the cytoplasmic components of the egg of *C. commercialis* show an increasing content of nucleic acid with decreasing size of granules, the ground cytoplasm containing the highest concentration of nucleic acid. Cleland's observations need to be corroborated, using the eggs of different species of oysters.

The formation and composition of yolk in the eggs of animals other than bivalves have been studied by many investigators, frequently with different and sometimes contradictory results. As Brachet (1944) stated nearly 20 years ago, the problem cannot be resolved at present. This uncertainty about yolk and other granules still persists and probably will continue until the ultrastructure of the marine egg is thoroughly explored by electron microscopy.

Examination with the light microscope of ripe, unfertilized, and unstained eggs of *C. virginica* discloses a multitude of tightly packed minute granules in the cytoplasm which obscure the inner portion of the egg. The granules appear to be uniformly distributed around the nucleus (fig.

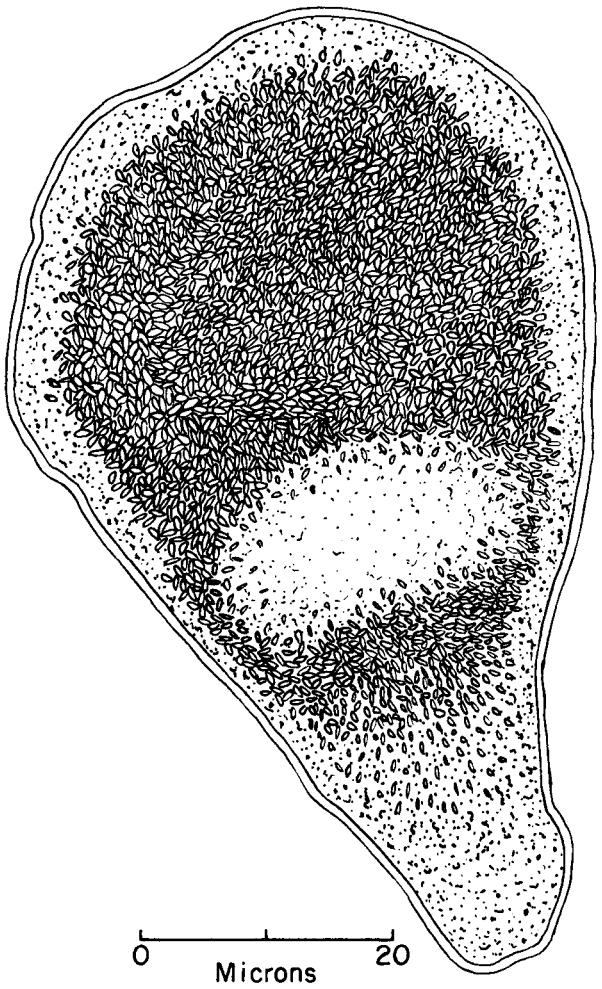

FIGURE 301.—Camera lucida drawing of live unfertilized egg of *C. virginica* in sea water. Germinal vesicle not visible under yolk granules.

301). The eggs are devoid of pigment. Oil globules of different sizes can be made visible under high magnification by gently pressing the egg under a coverslip; by using fat-staining dyes (Sudan II, III, or Black Sudan B) they become conspicuous (fig. 303). Under the effect of dye (dissolved in weak alcohol) the small granules of lipid yolk, stained dark red or black, gradually fuse into large globules and penetrate the vitelline membrane and a slight pressure will force them through it (fig. 304). The size of the globules increases during the time that the preparation remains under the microscope. These artifacts are due to the fusion of globules under the effect of dye.

The mitochondria of *C. virginica* can be stained by a 0.5 percent solution of Janus green in sea water. They appear as small rodlike structures uniformly distributed in the subcortical layer of the egg (fig. 305). The origin of fatty or lipid yolk in *C. virginica* has not been studied. In *Mytilus* eggs the lipid of the yolk apparently arises in an intimate association with the Golgi apparatus (Worley, 1944). In *Lymnaea* (Bretschneider and Raven, 1954) they are formed in certain parts of the protoplasm independently of cell structures visible under the light microscope.

In the eggs of the Bombay oyster, *C. cucullata*, which are similar to those of *C. virginica*, the fatty yolk, according to Rai (1930), is formed directly from the Golgi vesicles. Mitochondria exist in the eggs of this species in the form of very minute granules forming a circumnuclear ring. Later they grow in size and are more or less uniformly distributed. This conclusion is in agreement with the observations of Gatenby and Woodger (1920), who found that in *Helix* and *Limnaea* the Golgi elements gradually spread throughout the ovocyte and probably take part in the formation of yolk bodies. They found no evidence that part of the mitochondrial constituents of cytoplasm metamorphose into yolk.

Oyster eggs placed for 5 minutes in a dilute solution (1 to 25,000 or 1 to 30,000) of toluidin blue 0 and washed in sea water are colored metachromatically. Pasteels and Mulnard (1957) found

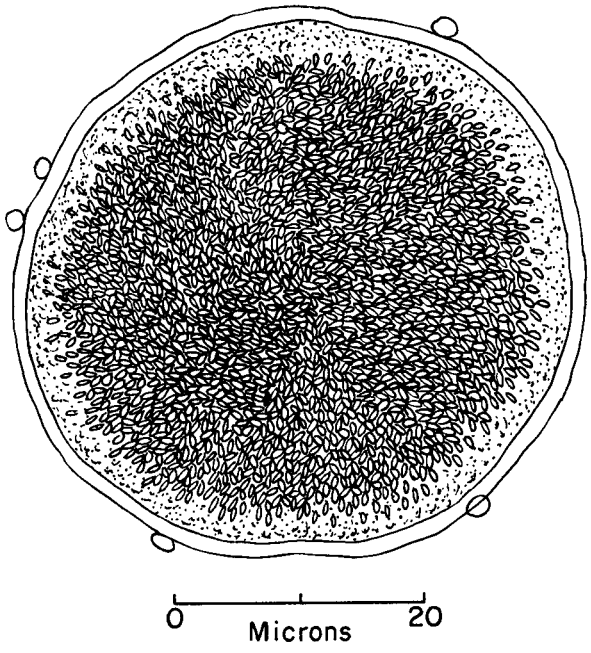

FIGURE 302.—Camera lucida drawing of live egg of *C. virginica* a few minutes after fertilization.

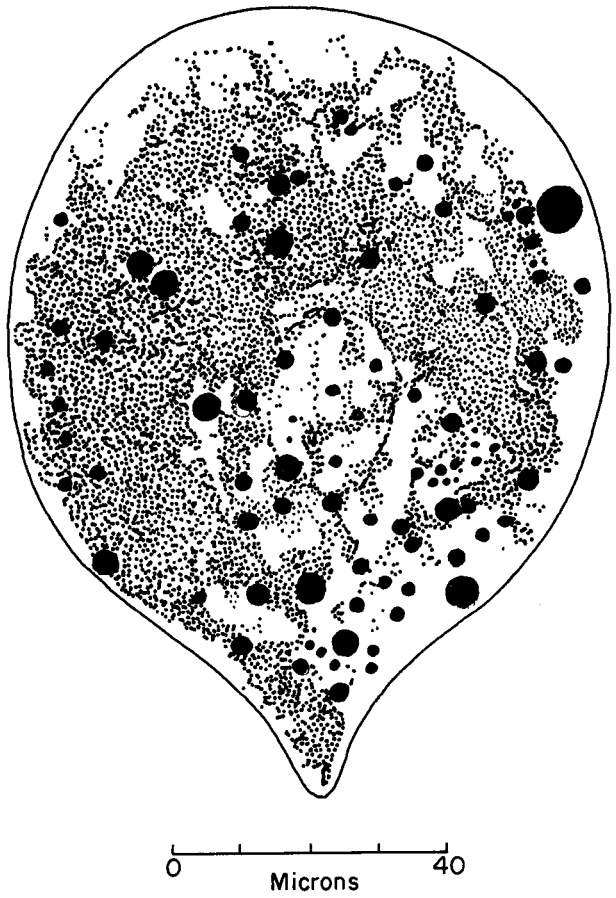

FIGURE 303.—Oil globules in the unfertilized egg of *C. virginica* after staining in Sudan III. Whole mount. Drawing made from a photomicrograph.

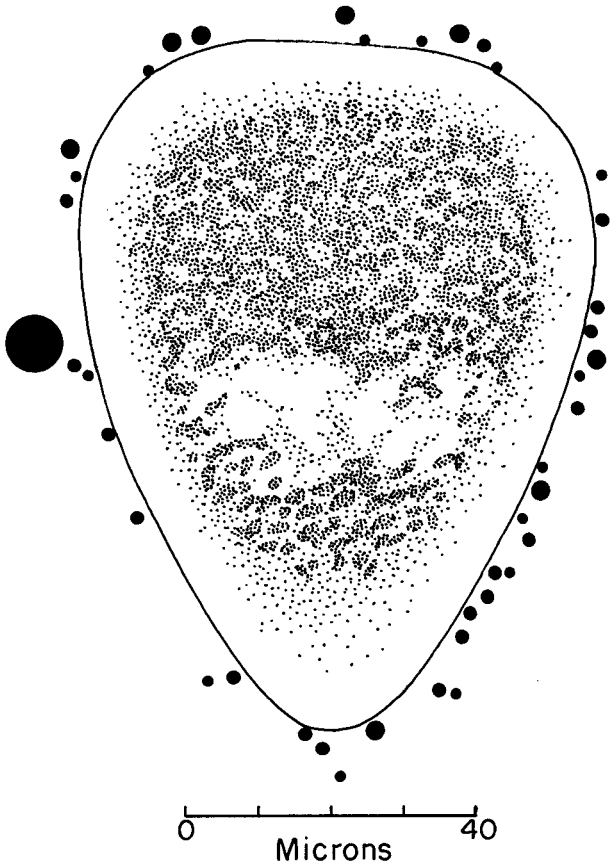

FIGURE 304.—Unfertilized egg of *C. virginica* stained with Black Sudan B; slight pressure on a coverslip forces the oil globules through the vitalline membrane. Drawn from life.

that the development of eggs of the Portuguese oyster, *C. angulata*, is not affected by toluidin blue used in such dilute solution for only a short time. The dye is fixed at the level of the small granules, which the cytologists designate as alpha granules, uniformly distributed in the cytoplasm between the yolk vesicles. Later in the development of a fertilized egg, new and larger granules, called beta granules, appear at the time of prophase. Their higher metachromasy is acquired at the expense of the alpha granules. Subsequent studies (Mulnard, Auclair, and Marsland, 1959) have suggested that the beta granules are related to the Golgi complexes of the eggs.

The alpha granules of the unfertilized eggs of *C. angulata* can be separated from the yolk vesicles by centrifuging; they are displaced in the direction of the centrifugal force (Pasteels and Mulnard, 1957), while the beta granules at the pronucleus stage of the fertilized egg are moved in the centripetal direction. The alpha and beta particles of Pasteels and Mulnard probably correspond to the P and L granules of Cleland. Personal observations show that in ripe but unfertilized eggs of New England *C. virginica* stained with toluidin blue, elements corresponding to the alpha particles of Pasteels assume a lavender color while mitochondria and other smaller granules are bluish. The nucleolus is also of bluish color. After 10 minutes of centrifuging at 4,000 times gravity the yolk granules of the stained eggs concentrate at the lower (centrifugal) pole, while the alpha particles of lavender hue and slightly bluish mitrochondria are at the opposite pole (fig. 306).

Metachromatic granules have been described in the eggs of various bivalves. They were found in *Barnea candida* (Pasteels and Mulnard, 1957); *Mactra* (Kostanecki, 1904, 1908; *Mercenaria* (*Venus*) *mercenaria*, *Mytilus edulis*, and *Spisula solidissima* (Worley, 1944; Kelly, 1954, 1956;